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published in
Journal of Biological Chemistry
2005

DOI (link to publisher)
10.1074/jbc.M414094200

document version
Publisher's PDF, also known as Version of record

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 13, Issue of April 1, pp. 12996–13003, 2005

Printed in U.S.A.

The Sec-independent Function of *Escherichia coli* YidC Is Evolutionary-conserved and Essential*

Received for publication, December 15, 2004, and in revised form, January 18, 2005
Published, JBC Papers in Press, January 25, 2005, DOI 10.1074/jbc.M414094200

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YidC plays a role in the integration and assembly of many (if not all) *Escherichia coli* inner membrane proteins. Strikingly, YidC operates in two distinct pathways: one associated with the Sec translocon that also mediates protein translocation across the inner membrane and one independent from the Sec translocon. YidC is homologous to Alb3 and Oxa1 that function in the integration of proteins into the thylakoid membrane of chloroplasts and inner membrane of mitochondria, respectively. Here, we have expressed the conserved region of yeast Oxa1 in a conditional *E. coli yidC* mutant. We find that Oxa1 restores growth upon depletion of YidC. Data obtained from *in vivo* protease protection assays and *in vitro* cross-linking and folding assays suggest that Oxa1 complements the insertion of Sec-independent proteins but is unable to take over the Sec-associated function of YidC. Together, our data indicate that the Sec-independent function of YidC is conserved and essential for cell growth.

Membrane proteins destined for the mitochondrial inner membrane, chloroplast thylakoid membrane, or plasma membrane of bacteria are targeted and inserted via distinct and partly conserved pathways (1–4). In *Escherichia coli*, two pathways have been identified by which membrane proteins integrate into the inner membrane. One pathway involves both the Sec translocon and YidC (the Sec-YidC pathway), whereas the other only involves YidC (the "YidC-only" pathway) (5, 6).

Most inner membrane proteins (IMPs)[1] appear to follow the Sec-YidC pathway. Targeting to the Sec translocon is mediated by the signal recognition particle (SRP) that associates with a hydrophobic targeting signal in a nascent IMP near the ribosomal exit site (7, 8). The nascent IMP is then transferred to the Sec translocon in the inner membrane. The mechanism of targeting and transfer is not fully understood but most likely involves both the SRP receptor FtsY that is in part associated with the inner membrane and the large ribosomal subunit that has affinity for the Sec translocon. The core of the Sec translocon is the heterotrimeric SecYEG complex that functions not only as the insertion site for IMPs but also as the general translocation channel for secretory proteins (9, 10). Translocation of secretory proteins and of large periplasmic domains of IMPs is powered by the cytosolic ATPase SecA that is connected with the Sec translocon during the translocation process. Cross-link and pull-down experiments have identified YidC as another Sec-associated component that interacts with Sec-dependent IMPs during their membrane insertion (11, 12). The contact with YidC is transient and specific for transmembrane segments (TMs) of substrate IMPs. The function of Sec-associated YidC is enigmatic and may be versatile. Order of interaction studies using nascent IMPs suggest a role for YidC both in the reception and the lateral diffusion of individual TMs from the Sec translocon into the lipid bilayer (13–15) as well as in assembly of multiple TMs near the Sec translocon (16). In support of this proposed function, membrane insertion of subunit a of F_1F_0-ATPase is affected upon depletion of YidC (17). Notably, YidC is not *per se* essential for insertion of all Sec-dependent IMPs, although it may enhance the fidelity and efficiency of this process (15, 18). A recent study indicates that YidC plays an important role in the folding of the Sec-dependent lactose permease (LacY) suggesting a function for YidC as a chaperone or folding catalyst (19).

In addition, YidC is involved in a Sec-independent integration process (20). Substrates of this YidC-only pathway include small phage coat proteins and the important endogenous IMP F_0c (subunit c of F_1F_0-ATPase) (reviewed in Ref. 4). Recent *in vitro* reconstitution experiments reveal a direct catalytic but otherwise undefined function of the Sec-independent form of YidC in membrane insertion (18, 21).

Interestingly, YidC is a member of the Alb3/Oxa1/YidC protein family (1–4). Alb3 and Oxa1 are involved in insertion of proteins into the chloroplast thylakoid and mitochondrial inner membrane, respectively. Alb3 is required for insertion of subunits of the light-harvesting chlorophyll-binding protein in an apparently Sec-independent process. However, association of Alb3 with SecY has been demonstrated in *Arabidopsis thaliana* (22), and Alb3 can functionally complement a conditional *E. coli yidC* mutant in both the Sec-YidC pathway as well as the YidC-only pathway suggesting the involvement of Alb3 in distinct insertion pathways similar to *E. coli* YidC (23).

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§ Supported by the Council for Chemical Sciences of the Netherlands Society for Scientific Research.

** Supported by National Institutes of Health Grant DK5113109.

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[1] The abbreviations used are: IMP, inner membrane protein; SRP, signal recognition particle; TM, transmembrane segment; HA, hemagglutinin; Lep, leader peptidase; IMV, inner membrane vesicle; IPTG, isopropyl 1-thio-β-D-galactopyranoside; mAb, monoclonal antibodies; PspA, phage shock protein A.

Oxa1 is the first identified and best studied member of this family (reviewed in Ref. 1). Oxa1 was found to mediate insertion and assembly of various proteins into the mitochondrial inner membrane without being absolutely essential for this process. Substrates of Oxa1 include both nuclear and mitochondrially encoded IMPs, most notably subunits of respiratory chain complexes. Cross-linking experiments revealed direct proximity between Oxa1 and its substrates early during their biogenesis and membrane integration as observed for *E. coli* YidC (24). Oxa1 has been purified as a homo-oligomeric, probably tetrameric, complex from *Neurospora crassa* mitochondria (25) consistent with the fact that mitochondria do not possess a Sec translocon (26). Based on these considerations, it has been postulated that Oxa1 and the Sec-independent form of YidC share structural and functional properties.

In the present study, we expressed the conserved region of yeast Oxa1 fused to a non-essential targeting domain of YidC in a conditional *E. coli yidC* mutant. The hybrid Oxa1 restores growth upon depletion of YidC and complements the insertion of Sec-independent proteins but appears incapable to take over the Sec-associated function of YidC. Thus, the Sec-independent function of YidC appears conserved and essential for cell growth.

EXPERIMENTAL PROCEDURES

Reagents and Sera—Restriction enzymes and the Expand Long Template PCR system were obtained from Roche Applied Science. T4 DNA ligase was from Invitrogen. The MEGAshortscript T7 transcription kit was from Ambion Inc. [^{35}S]methionine and protein A-Sepharose were from Amersham Biosciences. All other chemicals were supplied by Sigma. Antiserum against the hemagglutinin (HA) tag was purchased from Sigma, and antisera against YidC, leader peptidase (Lep), trigger factor, and OmpA have been described previously (11, 27).

Strains, Plasmids, and Growth Conditions—E. coli strain Top10F′ (Invitrogen) was used for cloning and maintenance of plasmid constructs. Strain MRE600 was used to prepare translation lysate for suppression of TAG stop codons in the presence of L-[3-(trifluoromethyl)-3-diazirin-3H-yl] phenylalanine ((Tmd)Phe)-tRNA^{sup} (11). Strain MC4100 was used to obtain inner membrane vesicles (IMVs) (prepared as described in Ref. 28). The YidC depletion strain JS7131 (20) was used for the preparation of IMVs and for *in vivo* protease accessibility experiments. The temperature-sensitive amber suppressor YidC depletion strain KO1672 and its isogenic parent strain KO1670 (29) were used for *in vivo* protease accessibility experiments. All strains were routinely grown in Luria Bertani (LB) medium with appropriate antibiotics.

Plasmids pC4Meth108FtsQTAG40, pC4Meth50LepTAG10, and pC4Meth50LepTAG15 have been described before (11, 14). Plasmid pEH1-ecOxa1His harbors the ecOxa1 fusion construct with a N-terminal hexahistidinyl tag under control of a *lac* promoter. For construction of this plasmid, the sequence encoding residues 2–247 of YidC was amplified by PCR from DNA isolated from *E. coli* DH5α. Codons for one methionine, one alanine, and six histidine residues were introduced via the 5′ primer directly upstream of the YidC sequence. The PCR product was cleaved by NcoI and KpnI and cloned into the expression plasmid pEH1 (30). The sequence encoding residues 43–402 of Oxa1 was amplified from genomic yeast DNA using an upstream primer that introduced a short linker containing a tobacco etch virus cleavage site. The resulting PCR product was digested with KpnI and SmaI and also cloned into the pEH1 plasmid, giving rise to plasmid pEH1-ecOxa1His. For complementation studies, a low copy ecOxa1 plasmid was constructed as follows. The EcoRV/AgeI fragment of pEH1-ecOxa1His was replaced by the corresponding fragment of pEH1-YidCX (lab collection), thereby removing the hexahistidinyl tag and introducing an XbaI site upstream of the YidC coding region. The hybrid construct lacking the tag was cloned XbaI/SmaI into pUC19, yielding pUC19-ecOxa1. Subsequently, the ecOxa1 coding sequence was cloned XbaI/SacI into the low copy expression plasmid pCL1921.Km, yielding pCL-ecOxa1. pCL1921.Km is a derivative of pCL1921 (31) containing the HincII fragment of pUC4K harboring the kanamycin resistance gene cloned into the NaeI site in the streptomycin resistance gene.

ecOxa1 expression plasmids were introduced into strain JS7131, KO1672, and KO1670 together with a compatible *LacI^q* plasmid (when indicated) to suppress uninduced expression of the ecOxa1 construct. For *in vivo* protease accessibility experiments, the previously described pASKIBA3-M13P2 and pBAD24-F_0cHA were used (27).

In Vivo Protease K Accessibility Assay—Strain JS7131 harboring pCL-ecOxa1 together with pASKIBA3-M13P2 was essentially grown as described (20). Briefly, cells were grown overnight in LB medium supplemented with 0.2% L-arabinose. The overnight culture was diluted 1:50 in fresh LB supplemented with 0.2% L-arabinose to express YidC. To deplete cells for YidC, L-arabinose was omitted from the medium, and for the expression of the ecOxa1 construct IPTG was added to the medium as indicated. Cells were grown to mid-log phase, harvested, and resuspended in M9 minimal medium. Expression of M13P2 was induced for 10 min by adding anhydrotetracycline (500 ng/ml). Subsequently, the cells were pulse-labeled with [^{35}S]methionine (30 μCi/ml), converted to spheroplasts and processed as described previously (32). For F_0c protease accessibility assays, the temperature sensitive YidC depletion strain KO1672 and its isogenic control strain KO1670 harboring pCL-ecOxa1 together with pBAD24-F_0cHA were grown as described (29). Briefly, both strains were grown overnight at 30 °C in LB medium. The overnight culture was diluted 1:50 into fresh LB. To deplete KO1672 for YidC, cells were grown at 42 °C, and for the expression of the ecOxa1 construct IPTG was added to the medium as indicated. Cells were grown to mid-log phase, harvested, and resuspended in M9 minimal medium. Expression of F_0cHA was induced for 10 min by adding L-arabinose (0.2%). Subsequently, the cells were pulse-labeled with [^{35}S]methionine (30 μCi/ml), converted to spheroplasts, and processed as described previously (32). As a control for depletion and expression, YidC and ecOxa1 levels were analyzed by SDS-PAGE and immunoblotting using YidC antiserum.

In Vitro Transcription, Translation, Targeting, and Cross-linking—Truncated mRNA was prepared as described (11) from HindIII linearized pC4Meth constructs. *In vitro* translation and cross-linking of nascent FtsQ and Lep derivatives carrying the photo-activable amino acid (Tmd)Phe were carried out as described (11). Targeting to IMVs and carbonate extraction have been described previously (11, 15). High salt sucrose pellet fractions and carbonate insoluble fractions were analyzed directly by SDS-PAGE and phosphorimaging.

Folding of in Vitro Synthesized lacY—In vitro transcription/translation/insertion was performed at 30 °C for 15 min, and IMVs containing *in vitro* synthesized LacY were collected onto 50% sucrose and washed with urea as described (33). Immunoprecipitation of the *in vitro* synthesized LacY was carried out as described previously (19) with minor modifications described below. After *in vitro* transcription/translation/insertion of LacY, IMVs were washed with 50 mM Tris-HCl (pH 7.5) and incubated with purified monoclonal antibodies (mAb) (25 μg/ml) as specified in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0 mM EDTA, 2% dodecyl-β-D-maltopyranoside for 6 h at 4 °C. Before the addition of mAb, 5% of each reaction mixture was saved for quantification of LacY (see Fig. 8, *Insertion*). Supernatants obtained by ultracentrifugation were treated with protein A-Sepharose beads for 2 h at 4 °C with continuous rotation. The beads were collected by a brief centrifugation, washed five times for 5 min at 4 °C with 100-fold protein A volumes of wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2% dodecyl-β-D-maltopyranoside], and then resuspended in 1% dodecyl-β-D-maltopyranoside and 1.0 mM dithiothreitol. Samples were kept at room temperature for 30 min and mixed occasionally. Samples were then analyzed as described (33). mAb4B1 and mAb4B11 were purified as described (19).

RESULTS

ecOxa1 Is Expressed in E. coli and Complements the Growth Defect of a Conditional yidC Strain—Members of the Alb3/Oxa1/YidC family are ubiquitous membrane proteins that share a similar topology with a conserved core of five TMs separated by relatively short loops (Fig. 1*A*). *E. coli* YidC has an additional TMs upstream from this core followed by a large periplasmic domain. The organellar Alb3 and Oxa have extended C-terminal domains downstream of the last TMs. Alb3 and Oxa are initially synthesized with an N-terminal targeting signal that is cleaved from the mature protein.

To study the function of Oxa1 in *E. coli*, a chimeric protein was constructed consisting of the first 247 amino acids of YidC fused to residues 43–402 of Oxa1 separated by a linker sequence at the fusion point (Fig. 1*B*). The fused N-terminal region of YidC comprises the first TMs and part of the periplasmic loop (Fig. 1*A*). The first TMs has been shown to function in

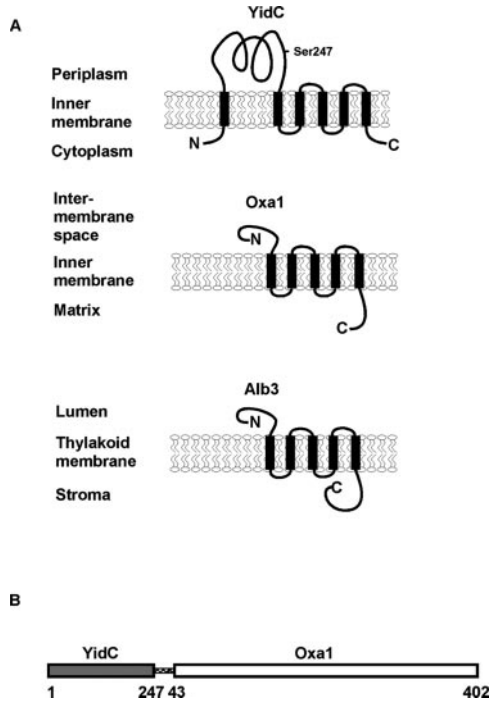

FIG. 1. *A*, membrane topology of Alb3/Oxa1/YidC family members. Oxa1 and Alb3 are initially synthesized with an N-terminal mitochondrial and chloroplast targeting sequence, respectively, that is proteolytically removed upon import. Mature Oxa1 and mature Alb3 are polytopic membrane proteins comprising five TMs. *E. coli* YidC is a polytopic membrane protein comprising six TMs, of which the first TMs functions as an uncleaved signal sequence. The position of amino acid 247 is indicated. *B*, schematic representation of the ecOxa1 fusion construct. The hybrid construct comprises the first 247 amino acids of YidC, a linker sequence (*hatched area*), and amino acids 43–402 of mature Oxa1.

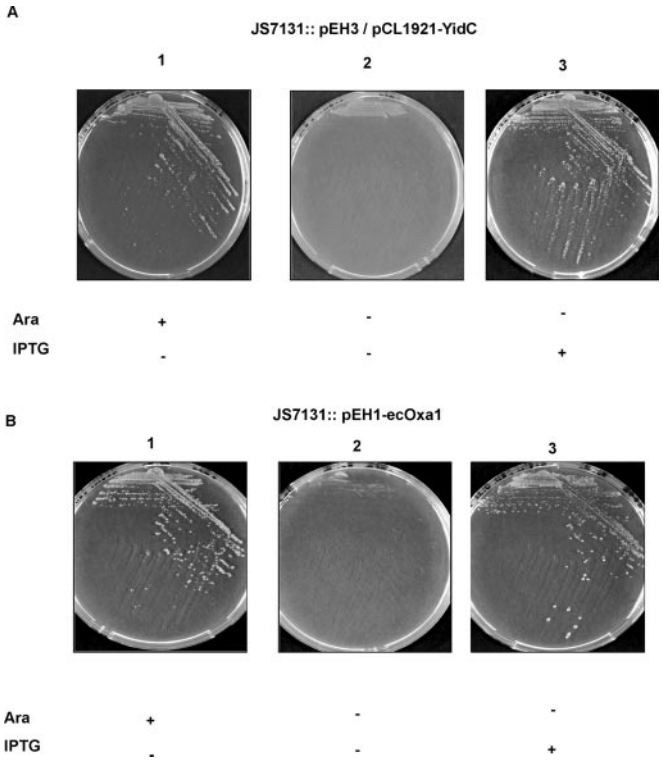

FIG. 2. **ecOxa1 complements growth in the YidC depletion strain JS7131.** *A*, JS7131 containing wild type *yidC* in the low copy expression plasmid pCL1920 was streaked on LB plates supplemented with 0.2% L-arabinose or 50 μM IPTG to induce expression of either the chromosomal *yidC* or the plasmid encoded *yidC*. To deplete cells for YidC, arabinose and IPTG were omitted. The plates were incubated overnight at 37 °C. *B*, JS7131 harboring ecOxa1 in the medium copy expression plasmid pEH1 was streaked and grown on LB plates as indicated in *A*.

the targeting of YidC to the inner membrane (34, 35). The periplasmic region downstream of the first TMs is neither conserved nor essential for YidC functioning *per se* (23, 36). The fused Oxa1 part consists of the conserved mature Oxa1, lacking its matrix targeting sequence. The hybrid construct was named ecOxa1 for *E. coli*-targeted Oxa1 and was cloned under *lac* promoter control in the medium and low copy expression plasmids pEH1 and a pCL1921 derivative, respectively (30, 31). The plasmids were introduced into strain JS7131 that depends on arabinose for YidC expression, and hence for growth. To minimize uninduced expression of the ecOxa1 construct in pCL1921.Km, a compatible *lacI*^{q} plasmid (pEH3) was used.

Complementation of the growth defect upon depletion of YidC was assayed by monitoring growth on solid medium in the absence of arabinose. As a control, JS7131 cells containing wild type *yidC* in the low copy vector pCL1921.Km (see "Experimental Procedures") grew only when IPTG was present in the medium to induce YidC expression thus validating our assay conditions (Fig. 2*A*, compare *panels 1* and *3*). The ecOxa1 construct expressed from the same low copy expression vector could not complement depletion of YidC under these conditions (data not shown). However, induced expression of the hybrid from the medium copy vector pEH1 did restore growth of JS7131 in the absence of arabinose (Fig. 2*B*, compare *panels 1* and *3*) indicating that Oxa1 can complement depletion of YidC.

To examine whether the plasmid-dependent complementation is related to a difference in expression levels, strain JS7131 harboring the various constructs was grown in liquid LB in the presence of IPTG or arabinose and analyzed by immunoblotting for ecOxa1 content (Fig. 3). The plasmids that were shown to complement the *yidC* conditional strain in Fig. 2 expressed comparable levels of YidC and ecOxa1 (IPTG-induced expression of pCL-YidC in the presence of pEH3 (*lane 5*) *versus* pEH1-ecOxa1 alone (*lane 6*)). Expression of ecOxa1 from the low copy plasmid pCL1921.Km could be enhanced to

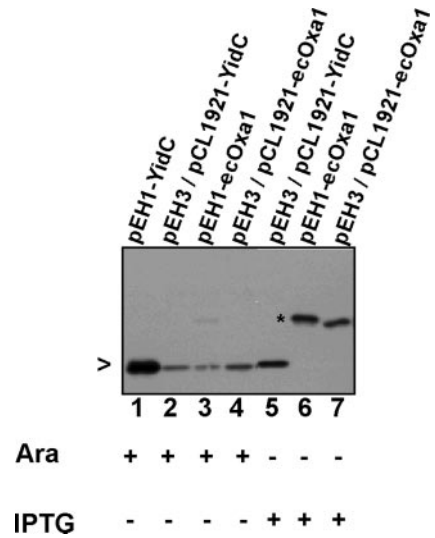

FIG. 3. **Expression of YidC and ecOxa1 in the YidC depletion strain JS7131.** Strain JS7131 harboring the indicated constructs was grown in LB medium in the presence of 0.2% L-arabinose (*lane 1–4*) or IPTG (50 μM, *lane 5–6*; 1 mM, *lane 7*). Cell samples were taken at mid-log phase, and 0.05 OD_{660} units of cells were analyzed by SDS-PAGE and immunoblotting for YidC and ecOxa1 content using YidC antiserum. The position of ecOxa1 is indicated by an *asterisk*, and the position of YidC is indicated by an *arrowhead*.

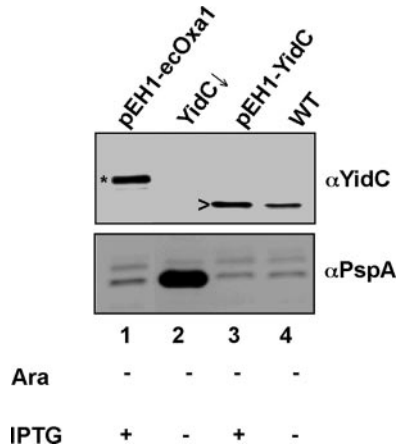

FIG. 4. **ecOxa1 is targeted to the inner membrane and suppresses the PspA response.** JS7131 cells expressing ecOxa1 or YidC were grown to mid-log phase in the presence of 30 μM IPTG (*lane 1*) or 20 μM IPTG (*lane 3*). Subsequently, IMVs were isolated by sucrose gradient centrifugation and analyzed by SDS-PAGE and immunoblotting using YidC or PspA antiserum. As controls, YidC levels and PspA levels were also analyzed in YidC depleted IMVs (*lane 2*) and IMVs isolated from wild type (*WT*) *E. coli* MC4100 (*lane 4*). The position of ecOxa1 is indicated by an *asterisk*, and the position of YidC is indicated by an *arrowhead*.

almost identical levels by using a higher concentration of IPTG (*lane 7*). Under the latter conditions, the growth defect upon depletion of YidC was restored but only on minimal medium plates (data not shown). Notably, under these conditions expression of ecOxa1 is higher than the arabinose-induced expression of YidC from the chromosomal *yidC* gene (*lane 2*) that yet fully supports cell growth in any tested medium. The difference in expression levels between wild type YidC and ecOxa1 was already observed in cells grown in the presence of arabinose but in the absence of IPTG. pEH1-YidC showed much stronger background expression than pEH1-ecOxa1 indicating a relatively low expression of the ecOxa1 construct (compare *lanes 1* and *3*) possibly caused by inefficient codon usage. Together, the data indicate a concentration-dependent complementation by the ecOxa1 construct of the growth defect in strain JS7131 caused by depletion of YidC.

ecOxa1 Is Localized in the Inner Membrane and Complements the Phage Shock Protein A (PspA) Response—To verify that ecOxa1 is correctly localized in the *E. coli* inner membrane, IMVs were gradient-purified from JS7131 cells expressing ecOxa1. As expected (23, 34, 35) and shown by immunoblotting using anti-YidC (Fig. 4, *lane 1*) and anti-Oxa1 serum (data not shown), the hybrid was co-purified with the IMVs indicative of its localization in the inner membrane.

We have shown that IMVs devoid of YidC contain massive amounts of PspA (Ref. 37; Fig. 4, compare *lanes 2* and *4*). PspA is a stress protein that responds to dissipation of the proton motive force, a consequence of YidC depletion caused by defects in the functional assembly of cytochrome *o* oxidase and the F_1F_0-ATPase complex (Ref. 37 and references therein). As shown in Fig. 4, the PspA response upon depletion of YidC is almost completely prevented by simultaneous expression of ecOxa1. This suggests that cells expressing ecOxa1 are able to sustain the proton motive force and that ecOxa1 complements the assembly of respiratory membrane complexes in *E. coli*.

ecOxa1 Can Fulfill the Sec-independent Membrane Integration Function of YidC—A subset of IMPs in *E. coli* integrates into the membrane via a Sec-independent mechanism that probably only involves YidC. To investigate whether ecOxa1 can replace YidC and facilitate the membrane integration of Sec-independent IMPs, we tested insertion and translocation of M13P2. M13P2 is an M13 procoat derivative extended at its C terminus with the P2 domain of Lep. M13P2 is synthesized with a signal sequence that is processed by Lep, resulting in mature M13P2 (Fig. 5*A*). Processing and membrane integration of M13P2 is, like wild type M13 procoat, strongly affected by YidC depletion but not by depletion of Sec components (32).

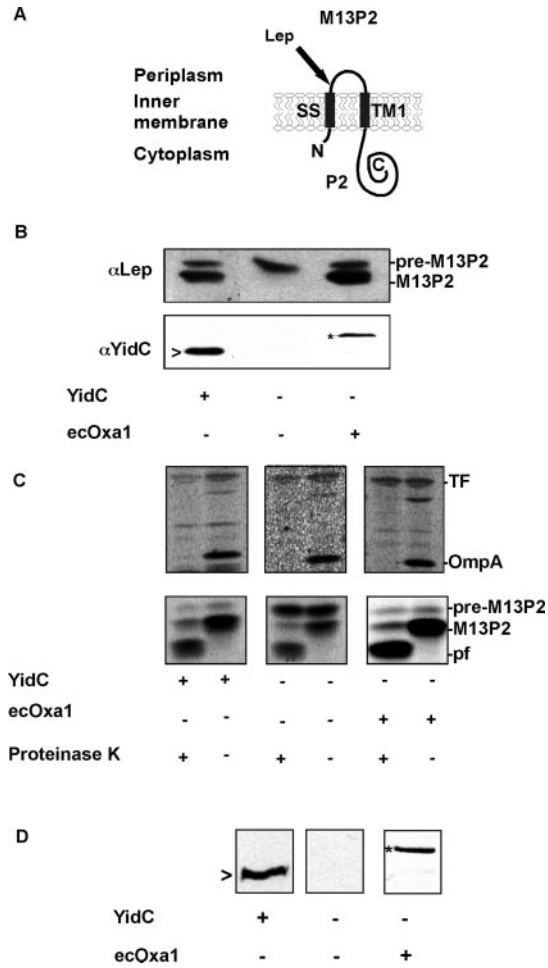

FIG. 5. **ecOxa1 promotes membrane insertion of M13P2.** *A*, membrane topology of the model protein M13P2. M13P2 is a M13 procoat derivative that is extended at its C terminus with the last 106 amino acids of the P2 domain of Lep. M13P2 is synthesized as precursor (pre-M13P2) with a signal sequence (*SS*) that is processed by Lep (*arrow*) to mature M13P2. *B*, steady state analysis of M13P2 processing in strain JS7131 harboring a low copy ecOxa1 expression plasmid. Cells were grown either in the presence of 0.2% L-arabinose (control cells) or 1 mM IPTG (ecOxa1 complemented cells). To deplete cells for YidC, arabinose and IPTG were omitted from the medium (YidC depleted cells). Expression of M13P2 was induced for 15 min, and M13P2 processing and YidC/ecOxa1 levels were analyzed in 0.1 OD_{660} units of cells by SDS-PAGE and immunoblotting using antibodies directed against the P2 domain of Lep and YidC, respectively. The position of YidC is indicated by an *arrowhead*, and the position of ecOxa1 is indicated by an *asterisk*. *C*, proteinase K accessibility assay to monitor translocation of the N terminus of M13P2 in strain JS7131 harboring a low copy ecOxa1 expression plasmid. Cells were grown as described in *B*, radiolabeled, converted to spheroplasts, and treated with proteinase K as described under "Experimental Procedures." The samples were immunoprecipitated using antibodies directed against the P2 domain of Lep (*bottom panels*) and antibodies against OmpA and trigger factor (*top panels*). OmpA (outer membrane protein) and trigger factor (*TF*, cytoplasmic protein) were used to monitor proteinase K treatment and spheroplast formation, respectively. Immunoprecipitated material was analyzed by SDS-PAGE and visualized by phosphorimaging. *pf*, proteolytic fragment of M13P2. *D*, expression analysis of YidC and ecOxa1 in cell samples used in *C*. 0.1 OD_{660} unit of cells was analyzed by immunoblotting using YidC antiserum. The position of YidC is indicated by an *arrowhead*, and the position of ecOxa1 is indicated by an *asterisk*.

Using immunoblot analysis under steady state conditions, we monitored processing of pre-M13P2 expressed from a plas-

mid in the conditional *yidC* strain JS7131 that also contained a compatible ecOxa1 expression plasmid. JS7131 cells harboring both plasmids were grown in the presence (control cells) or absence (YidC depleted cells) of arabinose or in the absence of arabinose combined with the presence of IPTG (ecOxa1 expressing cells). In control cells, most pre-M13P2 is converted into the mature form, whereas processing is completely inhibited upon depletion of YidC, as expected (Fig. 5*B*, Ref. 32). Expression of ecOxa1 restored processing of pre-M13P2 to the level of control cells arguing that ecOxa1 can mediate insertion of at least the signal peptide of M13P2 in a conformation that is suited for cleavage by Lep.

To examine membrane assembly of M13P2 in more detail, a protease accessibility assay was conducted using the YidC/ecOxa1 expression regime described above. Cells were pulse-labeled, converted to spheroplasts, and treated with proteinase K to degrade external (periplasmic) protein (domains). In spheroplasts derived from control cells, the periplasmic N-terminal tail of mature M13P2 was degraded by proteinase K, resulting in a smaller proteolytic fragment of distinct size (Fig. 5*C*, *pf*). OmpA and trigger factor (*TF*) are outer membrane and cytoplasmic control proteins used to monitor spheroplast formation and proteinase K treatment, respectively. Upon depletion of YidC, processing and protease accessibility of M13P2 were severely affected as shown before (Fig. 5*C*; Ref. 32). The accumulated pre-M13P2 was degraded in detergent-solubilized samples (data not shown) demonstrating that this form is not intrinsically proteinase K resistant but rather does not cross the inner membrane under these conditions. Notably, the defect in processing appeared less prominent than under steady state conditions (Fig. 5*B*), which might be caused by a difference in expression of pre-M13P2 under these conditions. In ecOxa1 expressing cells, processing of pre-M13P2 was restored and mature M13P2 was degraded by proteinase K to a similar extent and size as in the control cells, indicating that M13P2 is efficiently and correctly inserted with a translocated (accessible) N terminus and a cytosolic (protected) P2-domain (Fig. 5*C*). Translocation of OmpA appeared unaffected in both YidC depleted and ecOxa1 complemented cells, demonstrating that indirect inactivation of the Sec translocon had not occurred under these conditions. To verify the YidC and ecOxa1 expression conditions, cell samples were taken just before labeling. YidC and ecOxa1 levels were analyzed by SDS-PAGE and immunoblotting in these samples using a YidC antiserum (Fig. 5*D*). Clearly, only ecOxa1 (indicated by an *asterisk*) is produced in cells grown in the absence of arabinose and induced with IPTG, whereas YidC (indicated by an *arrowhead*) is only detected when cells are grown in the presence of arabinose.

As a second representative of this YidC-only integration pathway we analyzed insertion of the F_0c subunit of the F_1F_0-ATPase in the same experimental setup, using the temperature-sensitive amber suppressor YidC depletion strain KO1672 along with its isogenic control strain KO1670. F_0c is a small, double spanning membrane protein with a short N and C terminus exposed to the periplasm (Fig. 6*A*). We and others have recently shown that this native *E. coli* IMP requires only YidC for membrane integration (17, 18, 27). To permit immunodetection in protease accessibility experiments, a HA tag has been added to the periplasmic C terminus of F_0c (27). It is shown in Fig. 6*B* that depletion of YidC severely affects the accessibility of the HA tag as shown before (27) indicating that F_0c integration is compromised. Accessibility of the tag is restored upon expression of ecOxa1 indicating that Oxa1 can support integration and topogenesis of F_0c consistent with the complementation of the PspA response under these conditions (see Fig. 4). Expression of YidC and ecOxa1 was verified in Fig. 6*C*. Together, these data show that ecOxa1 mediates correct and efficient insertion of YidC-dependent/Sec-independent IMPs reminiscent of the role of Oxa1 in membrane protein insertion in mitochondria that lack a Sec translocon.

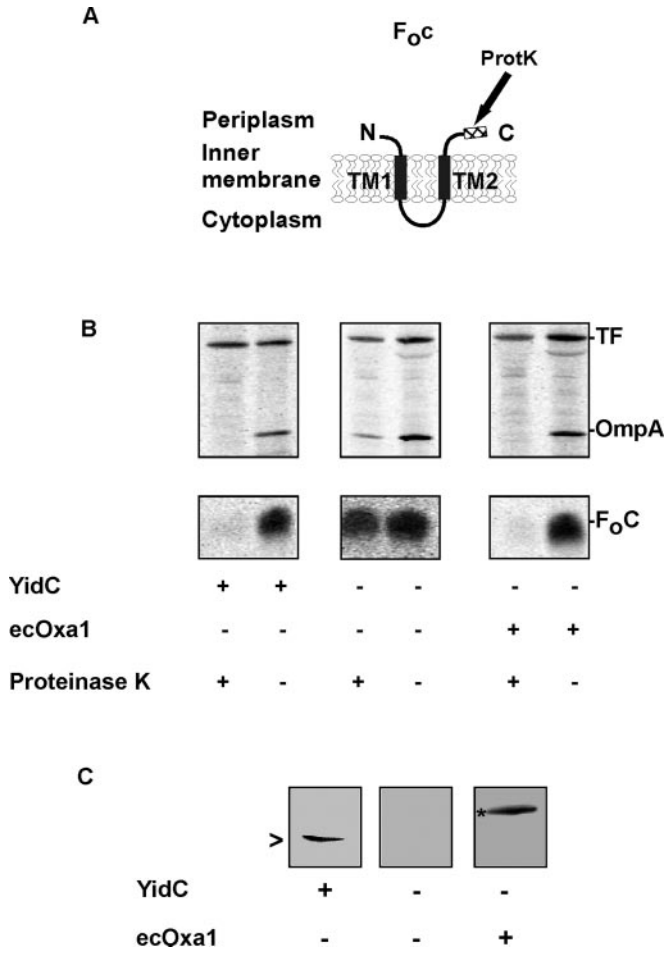

FIG. 6. **ecOxa1 promotes membrane insertion of F_0c.** *A*, membrane topology of the model protein F_0c, a small double spanning membrane protein with a short N and C terminus exposed to the periplasm. To facilitate detection of F_0c an HA tag has been attached to the C terminus that is accessible to proteinase K (*arrow*) in spheroplasts. *B*, proteinase K accessibility assay to monitor translocation of the C-terminal HA tag of F_0c in the temperature sensitive amber suppressor YidC depletion strain KO1672 and its isogenic control strain KO1670 harboring a low copy ecOxa1 expression plasmid. To deplete KO1672 for YidC, cells were grown at 42 °C to mid log-phase (YidC depleted cells). IPTG (1 mM) was added to induce expression of ecOxa1 (ecOxa1 complemented cells). Samples were prepared and processed as described under Fig. 5*C*. Anti-HA serum was used to immunoprecipitate F_0cHA. *C*, expression analysis of YidC and ecOxa1 in cell samples used in *B*, prepared and analyzed as described in Fig. 5*D*.

ecOxa1 Is Not Cross-linked to Nascent Sec-dependent Membrane Proteins—Next, we addressed whether ecOxa1 is capable of functioning in the Sec-YidC pathway of protein insertion. First, we investigated direct physical contacts of substrates of this integration pathway that are trapped during integration in the membrane. To monitor interactions we used an *in vitro* site-specific photo cross-linking approach that was previously applied to demonstrate the proximity of YidC to a nascent Sec-dependent IMP, FtsQ (11). Truncated FtsQ was synthesized to a length of 108 amino acids with a photo cross-linking probe incorporated at position 40 in the exposed signal anchor sequence (Fig. 7*A*), and IMVs were added to allow membrane integration of the translation intermediate. Subsequently, cross-linking was induced by UV irradiation, and membrane integrated material was recovered by extraction with sodium

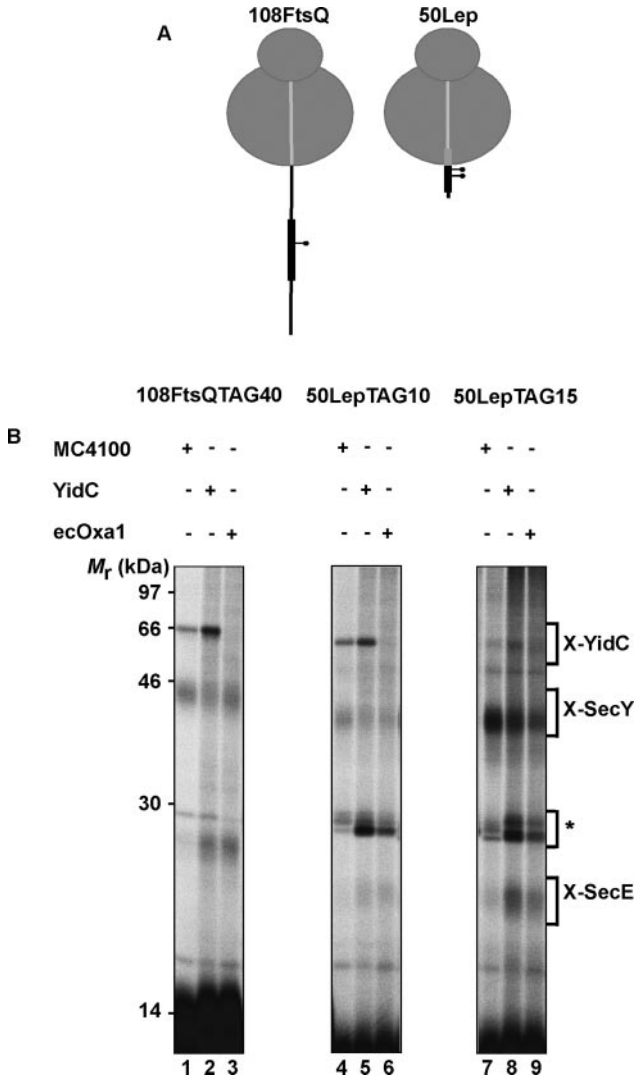

FIG. 7. **ecOxa1 is not in close proximity to nascent membrane inserted FtsQ and Lep.** *A*, schematic representation of the FtsQ 108mer and the Lep 50-mer with photo cross-linking probes at positions 40 (108FtsQ) and 10 or 15 (50Lep). The TMs are presented as *thick lines*. *B*, *in vitro* translation of nascent 108FtsQTAG40, 50LepTAG10, and 50LepTAG15 constructs was carried out in the presence of (Tmd)Phe-tRNA^{sup} and IMVs derived from a wild type *E. coli* strain (MC4100), a YidC overexpressing strain (YidC), or a strain depleted for YidC but expressing the ecOxa1 (ecOxa1) as indicated. YidC and ecOxa1 content of the IMVs used is shown in Fig. 4, *top panel*. After translation, samples were kept in the dark or irradiated with UV light to induce cross-linking and extracted with sodium carbonate to recover membrane inserted material. The pellet fractions were analyzed by SDS-PAGE and visualized by phosphorimaging. Pellet fractions from UV-irradiated samples are shown. The position of peptidyl tRNA is indicated by an *asterisk*.

carbonate. The IMVs used were derived from wild type cells, cells that overexpress YidC, and cells depleted for YidC but expressing ecOxa1 (see Fig. 4 for YidC and ecOxa1 content of the IMVs used). As shown before (11), 108FtsQTAG40 was cross-linked to YidC (Fig. 7*B*, *lane 1*; enhanced upon overproduction of YidC, *lane 2*) and to SecY. Using IMVs that contain ecOxa1 instead of YidC, no cross-linking product was detected in the ~70-kDa region (Fig. 7*B*, *lane 3*) indicating that YidC is effectively depleted and that ecOxa1 is not close to the inserting FtsQ TMs. Importantly, cross-linking to SecY was not affected under these latter conditions confirming previous data that showed that the initial insertion of nascent Sec-dependent proteins does not require YidC (15).

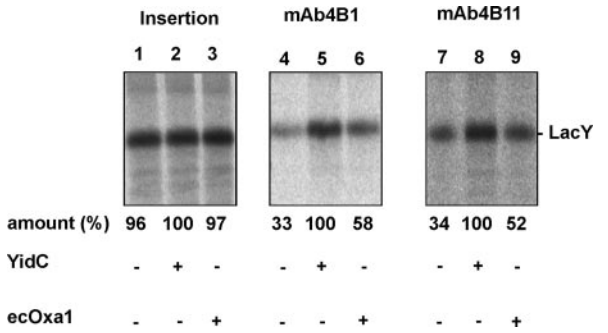

FIG. 8. **ecOxa1 does not support the folding of LacY.** *In vitro* synthesis and co-translational insertion of LacY into YidC depleted IMVs (*lane 1*), wild type IMVs (*lane 2*), or YidC depleted/ecOxa1 proficient IMVs (*lane 3*). After translation and insertion samples were extracted with urea. Folding of LacY was assayed by immunoprecipitation of membrane inserted LacY with monoclonal antibody mAb4B1 (*lanes 4–6*) that binds to periplasmic loop VII/VIII or mAb4B11 (*lanes 7–9*) that recognizes a discontinuous epitope containing determinants from cytoplasmic loops VIII/IX and X/XI. Quantification was approximated by measuring the density of the bands by phosphorimaging.

The absence of cross-linking of ecOxa1 to 108FtsTAG40 suggests that ecOxa1 is not near the insertion site for the FtsQ TMs in the Sec translocon. However, the lack of photo cross-linking could also be caused by a different orientation of the FtsQ TMs in the ecOxa1 containing IMVs. Therefore we decided to analyze a second representative of this Sec-YidC integration pathway, Lep, for its molecular environment during membrane integration using the same cross-linking approach. Interactions of nascent Lep derivatives have been studied in great detail providing profound knowledge of the initial stages of membrane insertion of Lep (13, 14). Here, we have analyzed cross-linking from positions 10 and 15 in the first TMs of 50Lep (Fig. 7*A*) that in wild type IMVs predominantly cross-link YidC and SecY, respectively, illustrating the oriented insertion of the TMs at this nascent chain length (Ref. 14; Fig. 7*B*, *lanes 4* and *7*}. Clearly, both 50LepTAG10 and -TAG15 constructs were not cross-linked to ecOxa1 suggesting that ecOxa1 is not in contact with TM1 of membrane inserted nascent Lep (Fig. 7*B*, *lanes 6* and *9*). Cross-linking of 50Lep TAG15 to SecY was less efficient in the ecOxa1 IMVs, which may reflect a somewhat altered orientation of TM1 in the YidC-free Sec translocon (Fig. 7*B*, compare *lanes 7* and *9*). Taken together, the cross-link data are consistent with the idea that ecOxa1 is unable to contact the TMs of nascent Sec-dependent IMPs during membrane integration.

ecOxa Does Not Support Folding of a Sec-dependent IMP—Recently, we have shown that depletion of YidC has little effect on the efficiency of membrane insertion of the Sec-dependent IMP LacY as seems true for all studied Sec-dependent IMPs (19). However, YidC appears to be required for folding of LacY into its correct three-dimensional structure (19). To examine whether ecOxa1 is able to complement functioning of YidC in folding of LacY we used our previously described *in vitro* assay (19). In this assay, co-translational insertion of *in vitro* synthesized LacY into IMVs is determined by ultracentrifugation of reaction mixtures treated with urea to remove LacY that is not inserted into the lipid bilayer. Moreover, LacY inserted into wild type IMVs *in vitro* is functional (33). Folding of membrane inserted LacY is assayed by analyzing recognition of two different conformational epitopes by specific monoclonal antibodies: mAb4B1 which binds to periplasmic loop VII/VIII, and mAb4B11, which binds to a discontinuous epitope containing determinants from cytoplasmic loops VIII/IX and X/XI (Ref. 19 and references therein).

As shown in Fig. 8, LacY is synthesized and inserted into wild type membranes, YidC depleted membranes, and YidC

depleted/ecOxa1 proficient membranes with approximately the same efficiency (*lanes 1–3*). Thus, according to the criteria used, the presence of YidC or ecOxa1 has no significant effect on membrane insertion of LacY. This again indirectly indicates that the Sec translocon is intact under these conditions. Importantly, both mAb4B1 and mAb4B11 are less efficient in immunoprecipitating membrane inserted LacY from YidC depleted membranes (*lanes 4*, *5*, *7*, and *8*) as observed before (19). The presence of ecOxa1 can only slightly reverse this effect (*lanes 6* and *9*) (see Fig. 4. for the YidC/ecOxa1 content of IMVs utilized). Apparently, Oxa1 is unable to efficiently substitute for YidC with respect to proper folding of the Sec-dependent LacY.

DISCUSSION

E. coli YidC is a member of the conserved Alb3/Oxa1/YidC protein family found throughout prokaryotes and eukaryotes (1–4). Family members constitute or contribute to a membrane insertion machinery that mediates insertion and assembly of hydrophobic membrane proteins into the inner membrane of bacteria and mitochondria and the thylakoid membrane of chloroplasts. *E. coli* YidC is in contact with the Sec translocon and associated with Sec-dependent IMPs during membrane insertion (11, 12). However, YidC is present in excess over the Sec translocon and the "free" YidC appears to constitute a separate "insertase" that is both necessary and sufficient for membrane insertion of a subset of Sec-independent IMPs (18, 20, 21). In contrast, mitochondria lack a Sec translocon (26), and Oxa1 most likely forms an homo-oligomeric insertion complex in the inner membrane (25).

In this study, we demonstrate that yeast Oxa1 (fused to the non-essential first TMs and periplasmic region of YidC, ecOxa1) can complement the growth defect that occurs upon depletion of YidC in a conditional strain. We found that this ecOxa1 can mediate insertion and translocation of two Sec-independent IMPs suggesting that Oxa1 is fully capable of fulfilling the Sec-independent function of YidC. In contrast, two independent assays suggest that ecOxa1 fails to function in connection with the Sec translocon. First, ecOxa1 is unable to replace YidC in the folding of LacY, a Sec-dependent IMP. Second, ecOxa1 is not cross-linked to two Sec-dependent IMPs during membrane insertion. Together the data suggest that the Sec-independent function of YidC is conserved and essential for growth.

Yeast Oxa1 functions in the assembly of respiratory chain complexes such as the cytochrome oxidase and ATPase complex in the mitochondrial inner membrane (reviewed in Ref. 1). Most likely, Oxa1 operates on its own. This implies that it combines a range of functions necessary for insertion and assembly of membrane proteins (complexes). Recently, it has been demonstrated that the matrix-exposed C terminus of Oxa1 is required for the co-translational targeting of mitochondrially encoded membrane proteins by interacting with mitochondrial ribosomes thus obviating the need for a targeting factor (such as the SRP) and a separate ribosome receptor (38, 39). In addition, Oxa1 must assist lipid partitioning of hydrophobic sequences in both nuclear and mitochondrially encoded membrane proteins. Finally, Oxa1 must translocate hydrophilic domains of membrane proteins and perhaps assist in the assembly of multisubunit complexes. How these diverse functions are realized and combined is unclear. It has been suggested that Oxa1 functions as a chaperone to accelerate and facilitate membrane integration without being absolutely essential for this process (40).

The fact that Oxa1 can complement bacterial growth in the absence of YidC is surprising at first sight but perhaps not totally unexpected. First, the replaced regions of Oxa1 and YidC are similar in sequence (although not dramatically so) and topology. Second, YidC is remarkably tolerant toward replacement even of conserved amino acid residues. In an extensive mutagenesis study none of the analyzed residues appeared to be absolutely essential for YidC functioning (36). Third, thylakoid Alb3 has been shown to functionally replace YidC *in vivo* using a comparable hybrid construct (23). Similarly, the *A. thaliana* protein ARTEMIS that contains an Alb3/Oxa1/YidC-like domain can partially take over the function of yeast Oxa1 (41). At second sight, mitochondria lack any of the known Sec components and do not possess an SRP or SRP receptor in contrast to bacteria and chloroplasts pointing to divergence in targeting and insertion mechanisms (26).

To confirm complementation of the Sec-independent function of YidC, we verified the ability of ecOxa1 to support insertion and translocation of two proteins diagnostic for this route. The hybrid M13P2 construct has been used before for this purpose (32, 42). It is strictly YidC-dependent but derived from phage M13 procoat, which obviously is not an authentic nor permanent resident of the *E. coli* inner membrane. The F_0c subunit of the F_1F_0-ATPase is a native *E. coli* protein that was recently shown to require only YidC for membrane insertion in three independent studies (17, 18, 27). The mechanism of targeting of the F_0c subunit to YidC is a controversial issue. SRP has been implicated (27), but other studies point to a spontaneous mechanism (17, 18). It remains to be determined whether the C-terminal ribosome-binding region of Oxa1 contributes to the targeting process in the complemented strain. Notably, the C-terminal region of YidC is neither conserved nor essential for cell viability and growth (36).

Complementation of the Sec-associated function of YidC has been investigated previously by monitoring the translocation of periplasmic domains of Sec-dependent IMPs such as Lep *in vivo* (15, 20, 23). In our view, this is not a dependable assay for Sec-dependent substrates because the observed effects are small and occur only upon prolonged depletion of YidC when secondary effects such as a diminished proton motive force and decreased levels of subunits of the Sec translocon cannot be excluded. Here, we used an unbiased *in vitro* cross-linking assay to demonstrate that ecOxa1 does not cross-link to two different Sec-dependent IMPs probed from a position and at a stage during insertion where cross-linking to YidC is eminent (Fig. 7). The simplest interpretation of this observation is that ecOxa1 is not near inserting nascent Sec-dependent IMPs because it does not associate with the *E. coli* Sec translocon at all. This property of YidC might have been lost concomitantly with the Sec translocon during the evolution of mitochondria from endosymbiotic bacteria.

The function of the Sec-associated form of YidC is enigmatic. As discussed above, YidC is not critical for the insertion of IMPs into the Sec translocon nor for the translocation of their periplasmic loops. Interestingly, it has been shown recently that YidC is required for folding of the 12 membrane-spanning Sec-dependent IMP LacY using conformation-specific monoclonal antibodies to pull down *in vitro* inserted LacY (19). Here, ecOxa1 appeared unable to restore efficient formation of the conformational epitopes in the absence of YidC, whereas insertion of LacY was unaffected indicative of an intact Sec translocon. This indicates that ecOxa1 is unable to assist folding of LacY in the same manner as YidC. Considering that folding of LacY has been shown to occur during translation and membrane insertion (33), it seems plausible that ecOxa1 is unable to receive TMs and support formation of the three-dimensional structure of LacY because of its physical separation from the Sec translocon.

Our data indicate that ecOxa1 can complement the Sec-independent but not the Sec-associated function of YidC. Inter-

estingly, in reciprocal experiments presented in the accompanying study by Preuss *et al.*, it was shown that YidC expressed in mitochondria can functionally replace Oxa1. However, for complementation the C-terminal ribosome-binding domain of Oxa1 had to be appended onto YidC, suggesting that this acquired function of Oxa1 is essential for functioning in mitochondria. Taken together, these data suggest that the function of YidC and Oxa1 is generally conserved, but nevertheless they are adapted to their specific needs.

Our data also imply that the Sec-associated function of YidC is not essential for *E. coli* growth. It should be noted that this general conclusion is based on both *in vitro* and *in vivo* assays but using only a limited number of IMPs. Conceivably, folding of Sec-dependent IMPs can proceed in the absence of YidC but at a slower pace, which may pose no major problem under standard laboratory growth conditions. The Sec-independent function of YidC may be more vital as it directly affects the levels of important membrane protein complexes such as the F_1F_0-ATPase and cytochrome *o* oxidase (37). It is expected that the ecOxa1 construct will prove an important tool to dissect the dual functioning of the versatile YidC protein in more detail.

Acknowledgments—We thank Corinne ten Hagen-Jongman and Gert-Jan Haan for technical support.

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